

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PHASE OF
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Asst. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

(Our Deposit Account No. 03-3975)

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: PM 270584 /MJL/GB/C372.07/U
M# /Client Ref.

From: Pillsbury Madison & Sutro LLP, IP Group:

Date: Monday, May 22, 2000

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application <u>PCT/GB98/03483</u> ↑ country code	2. International Filing Date 23 November 1998 Day MONTH Year	3. Earliest Priority Date Claimed 21 November 1997 Day MONTH Year (use item 2 if no earlier priority)
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4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is May 21, 2000 = Sunday
May 22, 2000 = Monday

5. Title of Invention IMPROVEMENTS IN OR RELATING TO DISPLACEMENT ASSAYS

Inventor(s) BADLEY, et al

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).
8. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:
- a. ☒ Request;
 - b. ☒ Abstract;
 - c. 31 pgs. Spec. and Claims;
 - d. 12 sheet(s) Drawing which are ☐ informal ☒ formal of size ☒ A4 ☐ 11"
9. ☒ A copy of the International Application has been transmitted by the International Bureau.
10. A translation of the International Application into English (35 U.S.C. 371(c)(2))
- a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
 - (3) _____ pgs. Spec. and Claims;
 - (4) _____ sheet(s) Drawing which are: ☐ informal ☐ formal of size ☐ A4 ☐ 11"
 - b. ☐ is not required, as the application was filed in English.
 - c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
 - d. ☐ Translation verification attached (not required now).

RE: USA National Filing of PCT/GB98/03483

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11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
- a. ☒ --This application is the national phase of international application PCT/GB98/03483 filed November 23, 1998 which designated the U.S.--
- b. ☐ --This application also claims the benefit of U.S. Provisional Application No. 60/____, filed ____.--
12. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., **before 18th month** from first priority date above in item 3, are transmitted herewith (file only if in English) including:
13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau
14. ☐ Translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of **claim amendments** made before 18th month, is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).
15. **A declaration of the inventor** (35 U.S.C. 371(c)(4))
- a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
- b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
16. **An International Search Report (ISR):**
- a. Was prepared by ☒ European Patent Office ☐ Japanese Patent Office ☐ Other
- b. ☒ has been transmitted by the international Bureau to PTO.
- c. ☒ copy herewith (2 pg(s).) ☒ plus Annex of family members (1 pg(s).).
17. **International Preliminary Examination Report (IPER):**
- a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
- b. ☒ copy herewith in English.
- c.1 ☒ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
- c.2 ☒ Specification/claim pages #28-30 claims #1-21
Dwg Sheets #
- d. ☐ Translation of Annex(es) to IPER (required by 30th month due date, or else annexed amendments will be considered canceled).
18. **Information Disclosure Statement** including:
- a. ☒ Attached Form PTO-1449 listing documents
- b. ☒ Attached copies of documents listed on Form PTO-1449
- c. ☒ A concise explanation of relevance of ISR references is given in the ISR.
19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.
20. ☐ Copy of Power to IA agent.
21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): __ sheet(s) per set: ☐ 1 set informal; ☐ Formal of size ☐ A4 ☐ 11"
22. ☐ __ (No.) **Verified Statement(s)** establishing "small entity" status under Rules 9 & 27
23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) EUROPE of:
- | | <u>Application No.</u> | <u>Filing Date</u> | | <u>Application No.</u> | <u>Filing Date</u> |
|-----|------------------------|--------------------------|-----|------------------------|--------------------|
| (1) | <u>97309409.7</u> | <u>November 21, 1997</u> | (2) | _____ | _____ |
| (3) | _____ | _____ | (4) | _____ | _____ |
| (5) | _____ | _____ | (6) | _____ | _____ |
- a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
- b. ☐ Copy of Form PCT/IB/304 attached.

RE: USA National Filing of PCT/GB98/03483

24. Attached:

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25. Preliminary Amendment:

25.5 Per Item 17.c2, cancel original pages #__, claims #__, Drawing Sheets #26. **Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:**
Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☒ 17, ☐ 25, ☐ 25.5 (hilitte)

Total Effective Claims	minus 20 =	x \$18/\$9	= \$0	966/967
Independent Claims	minus 3 =	x \$78/\$39	= \$0	964/965
If any proper (ignore improper) Multiple Dependent claim is present,		add \$260/\$130	+0	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ BASIC FEE REQUIRED, NOW →→→→A. If country code letters in item 1 are not "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

See item 16 re:

1. Search Report was <u>not</u> prepared by EPO or JPO -----	add \$970/\$485	960/961
2. Search Report was prepared by EPO or JPO -----	add \$840/\$420 +840	970/971

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

→ <input type="checkbox"/> B. If <u>USPTO</u> did not issue <u>both</u> International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), -----	add \$970/\$485	+0	960/961
(only) → <input type="checkbox"/> C. If <u>USPTO</u> issued ISR but not IPER (or box 4(a) above is X'd), -----	add \$690/\$345	+0	958/959
(one) → <input type="checkbox"/> D. If <u>USPTO</u> issued IPER but IPER Sec. V boxes <u>not all</u> 3 YES, -----	add \$670/\$335	+0	956/957
(of) → <input type="checkbox"/> E. If international preliminary examination fee was paid to <u>USPTO</u> and Rules 492(a)(4) and 496(b) <u>satisfied</u> (IPER Sec. V <u>all</u> 3 boxes YES for <u>all</u> claims), -----	add \$96/\$48	+0	962/963
(these) 4) →			
(boxes)			

27.		SUBTOTAL =	\$840	
28.	If Assignment box 19 above is X'd, add Assignment Recording fee of ----\$40	+0		(581)
29.	Attached is a check to cover the -----	TOTAL FEES	\$840	

Our Deposit Account No. 03-3975

Our Order No. 41301

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C#

M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filedPillsbury Madison & Sutro LLP
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09/554956

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

BADLEY ET AL

Serial No.: New Application

Group Art Unit: Unknown

Filed: May 22, 2000

Examiner: Unknown

Title: IMPROVEMENTS IN OR
RELATING TO DISPLACEMENT
ASSAYS

PTO/PCT Rec'd 11 JUL 2000

July 11, 2000

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Please amend the above application as follows:

IN THE CLAIMS

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "any one of claims 1, 2 or 3" and insert
-- claim 1 --.

Claims 5, 6, 7, 9, 10, 11, 13, 15, 16 and 17, line 1 of each, delete "any
one of the preceding claims" and insert -- claim 1 --.

Claim 8, line 1, change "8" to -- 7 --.

Claim 12, line 1, delete "any one of claims 1-10" and insert -- claim 1 --.

Claim 14, line 1, delete "any one of claims 1-12" and insert -- claim 1 --.

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Claim 19, line 1, delete "or 18".

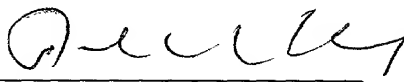
Cancel claims 20 and 21 without prejudice.

REMARKS

The claims have been amended above to improve form and to reduce the number of claims for consideration.

Respectfully submitted,

Pillsbury Madison & Sutro, LLP

By 

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12) PR+9
526 Rec'd PCT/PTO 22 MAY 2000
1Improvements in or Relating to Displacement Assays**Field of the Invention**

This invention relates, *inter alia*, to a method of performing an assay to detect the presence of a substance of interest in a sample, and to apparatus for performing the method.

Background of the Invention

Various assays for detecting the presence and/or amount of a substance of interest in a sample are known. One particular format of assay is the "displacement" assay in which, conventionally, the presence of an analyte of interest in a sample causes the displacement of a labelled substance (either a labelled binding partner or a labelled ligand) from a pre-existing binding partner/ligand complex. Generally speaking the amount of displaced labelled substance will be proportional to the concentration of the analyte of interest. Alternatively, one may employ "competition" assays, in which there is competition between the analyte of interest and a labelled competitor (such as labelled analyte or analogue) for binding to a limiting number of binding sites.

One particular assay is disclosed in WO 91/05262 (University of Michigan), which relates to an assay device, typically comprising a disposable test strip, wherein the presence of an analyte of interest in a test solution with which the test device is contacted, is indicated by a signal (such as a colour) at a distinct location on the device, and the absence of analyte is indicated by the same signal appearing at a different location.

As disclosed in WO91/05262 a first binding means (such as an immobilised antibody) is provided at one location on the test stick, and has binding specificity for the analyte of interest. A "heterobifunctional complex", comprising the analyte covalently coupled to a "signal generating molecule", is reversibly bound to the first binding means. In the presence of free analyte of interest in the sample, heterobifunctional complex is released from the first binding means and transported via "fluid conducting means" (typically a capillary test stick) to a second binding means, having binding specificity for the signal

generating molecule, such that the complex is captured. Typically the signal generating molecule is an enzyme (eg. horseradish peroxidase) such that, when the test stick is dipped into appropriate substrate solution, coloured product (the "signal") forms at the first binding means if no analyte was present in the test sample, or at the second binding means if analyte was present. The signal generating molecule may also be a fluorescent molecule or ultraviolet-absorbing agent.

In this sort of device, there is no interaction between the signal generating molecule and the binding means, beyond simple capture of the complex comprising the signal generating molecule. Thus, the signal generating molecule (the enzyme) is inherently capable of generating a signal (coloured product) whether or not it is released from the first binding means, and the act of capture of the signal generating molecule plays no part in the process of signal generation.

One relatively recent development in relation to assays is the use of devices which employ surface plasmon resonance (SPR). This phenomenon has now been described in several publications and is the basis of evanescent wave biosensors (for a review, see Hutchinson 1995, Molecular Biotechnology 3, 47-54, and references therein).

In summary, light incident on an interface between two media of different refractive indices will, at a specific angle of incidence, generate a resonant "evanescent" wave. The resonance is extremely sensitive to changes in the refractive index of the media. A change in the refractive index causes resonance to occur at a new angle of incidence. The change in refractive index is caused by mass binding to a thin (typically gold) film at the interface between the two media: the change in refractive index is proportional to the mass bound to the gold film.

Examples of such devices which utilise the evanescent wave phenomenon include the BIA lite™ and BIA core™ machines sold by Biacore AB (Sweden), the IA Sys™ device sold by Fisons (UK), and the BIOS-1 device sold by Artificial Sensor Instruments (Zurich, Switzerland).

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WO 92/18867 discloses an assay for an analyte of interest, the assay involving the displacement of a second reagent from a first reagent immobilised at a displacement surface, said displacement occurring in response to the presence of the analyte of interest, the displaced second reagent then causing a reaction at a detection surface which alters a third reagent immobilised in the detection surface, and wherein the alteration of the third reagent is detected by means of SPR.

WO 92/18867 is primarily concerned with the assay of analytes (such as haptens) of low molecular weight (page 1, lines 4-6) and addresses a lack of sensitivity in assaying such analytes which is apparent in the prior art (page 2, lines 1-17).

Typically, in WO 92/18867 the first reagent is an analogue of the analyte of interest, and the second reagent is an antibody specific for the analyte. Alternatively, the first reagent is an antibody specific for the analyte and the second reagent is a conjugate comprising an analogue (of the low molecular weight analyte) and a high molecular weight protein. The prior art teaches (page 3, lines 6-10) that:

"An analogue of the analyte is a substance which competes with the analyte for binding to a specific binder thereof. Often the analogue structure will be arranged to be as near as possible or even completely identical to the analyte".

Accordingly WO 92/18867 teaches that the analogue and the analyte should be structurally the same as "near as possible", and there is no disclosure or suggestion that there should be a difference in the relative binding affinities between the first reagent, the second reagent, and the analyte of interest.

Summary of the Invention

In a first aspect the invention provides a method of detecting the presence of an analyte of interest in the sample, the method comprising the steps of: providing a first surface having reversibly immobilised thereon a displaceable moiety; exposing the first surface to a sample comprising the analyte of interest, the analyte of interest specifically displacing the displaceable moiety from the first surface; causing the displaceable moiety displaced

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from the first surface to contact a second surface bearing a capture moiety which specifically binds to the displaceable moiety, so as to capture the displaceable moiety on the second surface, said capture generating a detectable signal; and detecting the signal, wherein said detection is performed by means other than surface plasmon resonance.

The method of the invention may be used to detect the presence of any analyte of interest. Examples of such analytes include antibodies (which may be useful in diagnostic methods), nucleic acids (DNA and/or RNA), hormones (of both the protein and the steroid variety), growth factors and the like. Of particular interest are sex and/or fertility hormones and analogues thereof, such as estrone-3-glucuronide (E3G), progesterone-3-glucuronide (P3G), human chorionic gonadotrophin (hCG), luteinising hormone (LH) and follicle stimulating hormone (FSH), wherein detection of such analytes may be useful in pregnancy testing. Conveniently the analyte is present in a liquid sample, especially a mammalian body fluid, such as blood, serum, urine or saliva.

The method of the invention may be used qualitatively (e.g. to detect the presence of the analyte of interest) or, more preferably, quantitatively such that the amount of analyte present may be determined.

The displaceable moiety and the capture moiety advantageously comprise members of a specific binding pair, such that when the displaceable moiety is displaced from the first surface it may readily be captured specifically at the second surface by the capture moiety. Many specific binding pairs are known to those skilled in the art and include, for example, antigens/antibodies, complementary (i.e. specific base pairing) nucleic acid strands, enzymes/substrates, lectins/sugars, hormones/receptors, and the like.

Generally the first surface will comprise a plurality of the displaceable moieties. Typically the displaceable moieties form a coating or layer attached to the first surface. The displaceable moiety may be directly attached to the first surface. However, more generally, the displaceable moiety will be indirectly attached to the first surface via an intervening molecule, which arrangement facilitates accomplishment of the desired degree of reversible immobilisation of the displaceable moiety.

Typically the intervening molecule will be immobilised to the first surface, by any of a number of conventional chemical techniques, whilst the displaceable moiety is relatively weakly attached to the intervening molecule (and thus reversibly immobilised upon the first surface). Desirably the intervening molecule will be an analogue (such as a mimotope, discussed below) of the analyte of interest, the arrangement being such that the binding affinity of the displaceable moiety (or, in other embodiments, of the intervening molecule) for the intervening molecule will be relatively low (eg. 1-50%, more typically 1-10%, preferably 1-5%) compared to the binding affinity of the displaceable moiety for the analyte of interest. Accordingly, in the presence of the analyte of interest, the analyte will tend specifically to displace the displaceable moiety from the first surface. Advantageously the method of the invention comprises specific, affinity-related displacement of the displaceable moiety, such that the analyte of interest causes quantitative displacement of the displaceable moiety. The relative affinities are desirably selected such that the presence of a small amount of analyte is sufficient to displace a reasonable amount of the displaceable moiety, so that the assay method of the invention is of high sensitivity.

In preferred embodiments the displaceable moiety comprises an immunoglobulin molecule or antigen-binding derivative thereof (such as a Fv, Fab, Fab₂, scFv, heavy chain variable regions ["Hcv"], such as those obtainable from llamas and camels] and the like). Particularly convenient examples are monoclonal antibodies, bispecific antibodies and "Diabodies", and fusion (chimeric) polypeptides comprising an immunoglobulin molecule or antigen-binding derivative thereof. Fusion proteins may comprise, for example, an immunoglobulin molecule (or antigen-binding derivative thereof) joined to one or more additional functional domains, such as an enzyme (e.g. catalase or glucose oxidase), or a portion which facilitates specific capture by the capture moiety (e.g. a mimotope - i.e. a structural analogue of an epitope recognised by an antibody, or some other molecule such as biotin, avidin or other member of a specific binding pair, wherein the capture moiety comprises the reciprocal member of the specific binding pair), or facilitates reversible immobilisation on the first surface.

In general, the capture moiety will comprise the reciprocal member of a specific binding

pair (i.e. the reciprocal member is that which binds to the member of the pair normally present in the displaceable moiety), and conveniently will be immobilised on the second surface. Conventional chemical methods, well known to those skilled in the art, may be used to effect such immobilisation. It will be understood that the term "immobilisation" in relation to the capture moiety, is intended to indicate that the capture moiety remains bound to the second surface under normal assay conditions, although when an assay has been completed harsh chemical treatment (e.g. very high or very low pH, or the use of chaotropic agents) might be possible to remove the capture moiety from the second surface, such that the surface may be coated with a different capture moiety, enabling performance of the method of the invention for a different analyte of interest. In general however, such treatment is not necessary as one can normally modify the first surface appropriately for use in connection with another analyte.

An important feature of the present invention is the nature of the second surface. In the present invention, the second surface takes what may be described as an "active" part in the signal generation, which contrasts with conventional prior art assays, in which the surface simply acts as in a passive manner as a support. In the present invention, it is the act of capture of the displacement moiety at the second surface which leads indirectly or (preferably) directly to the generation of the detectable signal. Generally the displaceable moiety cannot generate the detectable signal which is actually observed or monitored in the assay unless and until it is captured at the second surface, and generally signal generation depends upon an interaction of the displaceable moiety with the second surface.

The present invention provides a number of advantages over prior art assay methods and devices. Firstly, the invention allows for the use of a generic assay method and device which can readily be modified for use in the detection of any analyte of interest: the same second surface can be used (with the same type of signal generated and detected), only the relatively cheap, simple first surface need be altered, so that the displaceable moiety is displaced in response to the presence of the appropriate analyte. Secondly, the assay method and device, incorporating a specific displacement step, allow for the testing of samples which are heterogeneous (e.g. include particulate matter such as food particles,

blood cells or bacteria) or crude and contain many contaminants (e.g. serum, urine etc.). In addition, the assay of the present invention includes both a specific displacement step, and a specific capture step. This means that the overall specificity of the assay is effectively the mathematical product of the specificities of the displacement and capture steps, giving a far higher specificity than that conferred by most prior art assays. Moreover, this is achievable in a format which, as far as the user is concerned, is essentially a one-step process: the person performing the assay simply has to contact the sample with the first surface, which sets in chain the series of events which lead to the generation of the detectable signal.

In certain embodiments the second surface forms the detection surface of an evanescent or acoustic wave-type biosensor (e.g. as disclosed in EP 0 416 730; EP 0 517 001; or WO 97/04314) such that capture of the displaceable moiety at the surface causes an increase in mass which generates a detectable change in the signal at the second surface, (i.e. capture of the displaceable moiety regulates or modifies a pre-existing signal in a detectable manner).

In some embodiments, capture of the displaceable moiety at the second surface inhibits, in a detectable manner, the generation of a signal, such inhibition constituting the "detectable signal". For example, capture of the displaceable moiety may inhibit a reaction on-going at the second surface: in one embodiment, the second surface comprises a plurality of enzyme molecules which catalyse a reaction producing a detectable (e.g. coloured) product, and the displaceable moiety comprises an immunoglobulin molecule with specific binding activity for the enzyme, such that binding of the displaceable moiety to the enzyme inhibits its catalytic activity (or modifies it in some other way e.g. by binding to an allosteric site), thus producing a detectable change in behaviour of the enzyme.

In alternative preferred embodiments, an evanescent or acoustic wave propagated at the second surface interacts with the displaceable moiety to generate an entirely new signal, rather than the modulation of a pre-existing signal. For example, the displaceable moiety may comprise a fluorophor which is stimulated by the evanescent wave at the second

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surface to generate fluorescence: because of the short "range" of the evanescent wave, the fluorophor cannot be stimulated unless the displaceable moiety is captured at the second surface. Further, a degree of specificity is involved in the interaction as the fluorophore and wavelength of the evanescent wave are selected such that the evanescent wave has appropriate characteristics to cause excitation of the fluorophor.

In a preferred embodiment, the capture of the displaceable moiety by the capture moiety modulates an electrochemical property of the capture moiety, which modulation comprises the detectable signal. Suitable methods and substances are disclosed in our co-pending European patent application No. 97309425.3. In particular, the capture moiety typically comprises an immunoglobulin molecule (preferably an antibody) or an antigen-binding derivative thereof, immobilised to an electrically conductive support, wherein binding of the displaceable moiety to the capture moiety alters an electrochemical property of the capture moiety, generating a signal which may be detected by, for example, amperometric, voltametric or coulometric means. Preferably the capture moiety and displaceable moiety are selected such that capture of the displaceable moiety directly alters an electrochemical property (e.g. a redox potential) of the capture moiety.

In some embodiments of the invention, the distance between the first surface and the second surface is very small (e.g. in the range $50\mu\text{m}$ to 5mm), so that simple diffusion of the displaceable moiety (such as a displaced antibody molecule) between the first and second surfaces is sufficient to provide reasonably rapid response times, where these are required. The distance between the first and second surface may be even smaller than that suggested above in devices which utilise "nanotechnology" principles: in such devices the gap may be as small as the size of a single molecule (e.g. the 5 to 50nm of a typical immunoglobulin molecule or bispecific antibody), in order to minimise the times of mass transport between the first and second surface.

In alternative embodiments, where diffusion of the displaceable moiety from the first to the second surface is not sufficient, it will be necessary to provide some sort of "motive force" to convey the displaceable moiety from the first surface to the second surface. This could be by capillary flow, or by bulk fluid flow (using a pump means) or, for example,

by electrostatic attraction between the displaceable moiety and the second surface.

The first and second surfaces may be provided on separate respective first and second solid supports, which facilitates the inclusion of filter means as described below. Alternatively, the first and second surfaces may be provided on a single solid support. The solid support may be substantially planar, or particulate, and may be porous or impermeable. Where both the first and second surfaces are provided on a single support, the surfaces may be present as distant portions of the support, or may be present as closely adjacent portions of the support (e.g. with one surface surrounded, in two dimensions, by the other surface, or with the two surfaces intimately interdigitated). Examples of particulate supports include beads comprising polymeric substances (e.g. polysaccharides such as dextran; or latex or other material) which are well known to those skilled in the art. Examples of substantially planar supports include the sensor chips used in connection with known evanescent or acoustic wave type biosensors.

It may be preferred in some embodiments to position a filter means between the first and second surface such that particulate materials, present in some samples, may be removed. This may be desirable as such material may have detrimental effects on the assay result (e.g. due to non-specific binding of particulate material at the second surface). Suitable micropore filter membranes are well-known to those skilled in the art.

In a second aspect the invention provides apparatus for performing the method of the first aspect, defined above. Generally the apparatus will comprise: a first surface, having reversibly immobilised thereon a displaceable moiety, wherein the displaceable moiety is displaced from the first surface in the presence of an analyte of interest; and a second surface bearing a capture moiety which specifically binds to the displaceable moiety.

The apparatus defined above may take the form of a relatively cheap, disposable or replaceable component (e.g. as part of a kit) for use in association with other components, such as a "permanent" signal detection means. Alternatively the apparatus of the invention may be provided as substantially a "one piece" device, comprising one or more of the following: fluid conducting means; signal detection means for detecting a signal generated

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in the presence of analyte of interest; and data processing means for processing data output from the signal detection means.

The fluid (preferably liquid) conducting means may perform one or more of the following functions: introduction of the fluid sample to the first surface; transport of any displaced moiety from the first surface to the second surface; and removal of waste fluid through the fluid conducting means. Motive means suitable for use with the fluid conducting means may comprise, for example, a rotary or a peristaltic pump.

Desirably the apparatus is adapted for use with analytes of interest which are suspended or dissolved in liquids (especially body fluids, such as blood, serum, saliva and the like).

In a third aspect the invention provides a method of detecting the presence of an analyte of interest in the sample, the method comprising the steps of: providing a first surface having a displaceable moiety reversibly immobilised thereon by means of an interaction with an intervening moiety between the first surface and the displaceable moiety; exposing the first surface to a sample comprising the analyte of interest, the analyte of interest specifically displacing the displaceable moiety from the first surface; causing the displaceable moiety displaced from the first surface to contact a second surface bearing a capture moiety which specifically binds to the displaceable moiety, so as to capture the displaceable moiety on the second surface, said capture generating a detectable signal; and detecting the signal; wherein the binding affinity of the displaceable moiety for the analyte of interest is greater than that of the displaceable moiety for the intervening moiety.

In a fourth aspect the invention provides a method of detecting the presence of an analyte of interest in the sample, the method comprising the steps of: providing a first surface having a displaceable moiety reversibly immobilised thereon by means of an interaction with an intervening moiety between the first surface and the displaceable moiety; exposing the first surface to a sample comprising the analyte of interest, the analyte of interest specifically displacing the displaceable moiety from the first surface; causing the displaceable moiety displaced from the first surface to contact a second surface bearing a capture moiety which specifically binds to the displaceable moiety, so as to capture the

displaceable moiety on the second surface, said capture generating a detectable signal; and detecting the signal; wherein the binding affinity of the intervening moiety for the analyte of interest is greater than that of the displaceable moiety for the intervening moiety.

With regard to the third and fourth aspects of the invention, it is preferred that the binding affinity of the interaction by which the displaceable moiety is reversibly immobilised on the first surface is only 1-10% (more preferably 1-5%) that of the interaction which tends to cause displacement of the displaceable moiety (i.e. the interaction between the displaceable moiety and the analyte of interest in the third aspect, or the interaction between the intervening moiety and the analyte of interest in the fourth aspect of the invention). The preferred features of the method of the third and fourth aspects of the invention will generally be the same as those of the method of the first aspect, insofar as the methods conform. In the third and fourth aspects of the invention, a preferred means of detecting the signal is by way of SPR, as disclosed in WO92/18867. The invention also provides apparatus for performing the method of the third or fourth aspects defined above.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figures 1 - 3, 6 and 10 are schematic illustrations of assay methods in accordance with the invention;

Figures 4, 5A, 5B, 7, and 11 are traces (arbitrary response units against time) showing SPR signal outputs;

Figures 8 and 12 are graphs of SPR response (arbitrary response units) against time (in seconds); and

Figure 9 is a graph (% displaceable moiety captured at second surface, against flow rate in μ l per minute), showing the effect of flow rate on the amount of displaceable moiety captured in one embodiment of the invention, and how at low flow rates 100% of displaceable moiety specifically displaced from the first surface can be captured at the

second surface.

Example 1

A simple embodiment illustrating the basic concept of the invention is illustrated schematically in Figure 1. In Figure 1, a displaceable moiety is reversibly immobilised at a first surface 12. In the embodiment shown in Figure 1, the displaceable moiety is a bi-specific antibody 10 and the reversible immobilisation is achieved by interaction between the bi-specific antibody 10 and an intervening molecule 14 immobilised, by conventional chemistry, on the first surface 12. For simplicity, only a single molecule 14, and a single specific bispecific antibody 10, are shown. In practice, the first surface 12 would normally be coated with a plurality of intervening molecules 14, and a plurality of bispecific antibody molecules 10 (each having the same binding specificities) would be reversibly immobilised to the surface 12. This reversible immobilisation of the displaceable moiety is such that, under normal assay conditions, and in the absence of the analyte of interest, the displaceable moiety remains attached to the first surface, but is specifically displaced therefrom in the presence of the analyte of interest.

One binding site 16 of the bispecific antibody has binding specificity for the analyte of interest 18. The intervening molecule 14 immobilised on the first surface 12 is a structural analogue of the analyte of interest 18, such that the binding site 16 of the bispecific antibody 10 binds relatively loosely to the molecule 14, thus reversibly immobilising the bispecific antibody 10 on the first surface 12. However, the binding affinity of the binding site 16 for the analogue 14 is much lower (typically 1-10%, preferably 1-5%) than the binding affinity for the analyte of interest 18. Accordingly, upon contacting the first surface 12 with a sample comprising the analyte of interest 18, the analyte 18 competes successfully with analogue 14 for binding to binding site 16, thus specifically displacing the bispecific antibody 10 from the first surface 12. The displaced bispecific antibody 10 is then allowed to contact a second surface 20.

Referring again to Figure 1, the second surface 20 is coated with a plurality of molecules, each of which constitutes a capture moiety 22. The binding site 16 of the bispecific

antibody 10 has binding specificity for the capture moiety 22. Accordingly, the bispecific antibody molecule 10 displaced from the first surface is captured at the second surface 20. This capture generates a detectable signal, which is monitored. In the embodiment illustrated in Figure 1, the second surface 20 forms part of an SPR sensor.

Generally, the signal is generated by an interaction between the captured displaceable moiety 10 and the second (capture) surface 20. For example, the second surface 20 may comprise part of an evanescent wave detection device: capture of the displaceable moiety 10 modifies the characteristics of an evanescent wave propagated at the surface 20, in such a way that a detectable signal is produced. Examples of such devices are those which utilise the technique of surface plasmon resonance (SPR) which are now known in the art, or acoustic wave detection systems, wherein the signal is generated by the increase in mass of material bound to the surface 20.

However, other detection systems may also be envisaged, in which a different sort of interaction occurs between the displaceable moiety (when captured) and the second surface. These include the use of electroactive surfaces, wherein the capture of the displaceable moiety by the capture moiety directly modulates the electrochemical properties of the capture moiety, which modulation comprises the detectable signal. Suitable methods and substances are disclosed in our co-pending application filed on even date herewith, under our reference C319/U.

In the embodiment illustrated in Figure 1, the bispecific antibody 10 remains attached to the analyte of interest 18 when it binds to the capture molecule 22 on the second surface 20. In mass-based detection systems (such as SPR devices) this might be an advantage, especially if the displaceable moiety has a very small mass of its own. However, the continued attachment of the analyte 18 to the displaceable moiety 10 might be disadvantageous in other situations, especially where the generation of the detectable signal is not mass-dependent.

Example 2

An alternative embodiment (partially illustrated schematically in Figure 2) is also envisaged wherein the nature of the displaceable moiety, and its mode of reversible immobilisation to the first surface, are different to the embodiment illustrated in Figure 1. Functionally comparable components are annotated in Figure 2 using the same reference numerals as in Figure 1.

In Figure 2, there is provided a displaceable moiety 10 reversibly immobilised on a first surface 12, by interaction with an intervening molecule (antibody 14) which is immobilised to the surface 12. Antibody 14 has a specific binding affinity for the analyte of interest 18. The displaceable moiety 10 consists of an antibody molecule fused or covalently coupled to a "mimotope" 16 - a small molecule (normally a peptide, but possibly also a sugar, steroid, nucleic acid or other chemical moiety which may be conjugated to an immunoglobulin) which is a structural analogue of the epitope bound by antibody 14. The mimotope 16 is such that the binding affinity of the antibody 14 for the mimotope 16 is only 1-10% (preferably 1-5%) that of the affinity for the analyte 18. Accordingly, in the presence of the analyte of interest 18, the displaceable moiety 10 is specifically displaced from the first surface 12. The displaceable moiety may then be captured at a second surface (not shown), typically by a capture molecule which recognises the binding site 16' of the antibody portion of the displaceable moiety 10, essentially as indicated in Figure 2. Conveniently the mimotope is a peptide and forms a fusion protein (encoded by a genetic fusion between sequences encoding an immunoglobulin and the peptide). Alternatively the mimotope may be joined to an immunoglobulin molecule (or antigen binding portion thereof) by any one of conventional chemical techniques (see, for example, "Bioconjugate Techniques", Hermanson, Academic Press, 1996).

An advantage of the embodiment illustrated in Figure 2 is that the displaceable moiety 10 when displaced from the first surface 12, consists in large part of a conventional antibody molecule and is not attached to the analyte of interest 18. The presence of the analyte of interest 18 at the second surface (as in the embodiment illustrated in Figure 1) may

sometimes interfere, in some embodiments, with signal generation.

Example 3

This example relates to the dissociation of human chorionic gonadotrophin (hCG) from an immobilised antibody fragment located at one surface and capture/detection by antibody at a second surface. Figure 3 schematically illustrates this detection principle. This example is performed using the Biacore AB Bialite™ fitted with an upgrade kit and employs the use of surface plasmon resonance.

Initially, a CM5 sensor chip was set up as follows:

- i. A new CM5 sensor chip was docked in the Bialite™ biosensor and equilibrated with HBS (HEPES buffered saline) running buffer. The temperature was maintained at 25°C and the flow rate set to 10 μ l/min.
- ii. The liquid flow was set to cross two flow cells, flow cell 1 "first surface" and flow cell 2 "second surface" in series. The dextran surface was activated using 1-ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) activating chemicals from the Biacore AB amine coupling kit by injecting the EDC/NHS mixture into the sample loop and loading 40 μ l. The activation was repeated and can be seen marked (a) in figure 4. The Bialite™ response at the first surface is shown as a thin line, whereas the response at the second surface is shown as a thick line. This line thickness identification is used throughout subsequent Bialite™ traces.
- iii. Following activation, 40 μ l of a 50 μ g/ml solution of single chain antibody fragment recognising hCG (scFv3299HisH2) was injected across flow cell 1. This was followed by injection of 40 μ l of a 50 μ g/ml solution of a whole antibody recognising hCG (3299) across flow cell 2 and a further injection of 40 μ l of a 50 μ g/ml solution of scFv3299HisH2 across flow cell 1. These coupling steps can be seen marked (b) in figure 4.

- iv. Unreacted sites on the CM5 sensor chip were blocked by two sequential injections of 40 μ l of a 1M solution of ethanolamine. These can be seen marked (c) in figure 4.

By way of explanation, the sequence of scFv3299 is as disclosed in WO96/27612 (wherein it is described as FvKC-II), but with the *Bst*EII-*Sac*I DNA linker fragment replaced with a *Bst*EII-*Sac*I fragment encoding a flexible linker yielding the sequence:

FR-4 VH linker FR-1 VL

TVTVSSggggsgggsgggsgDIELT (seq: ID: No: 1)

The DNA sequence encoding the linker is described by Jackson *et al* ("Selection of variants of antibodies and other protein molecules using display on the surface of bacteriophage fd" in Protein Engineering: A Practical Approach, eds. Rees, Sternberg and Wetzel, publ. IRL Press, Oxford, 1992). It will be appreciated that numerous other anti-hCG antibodies would be equally suitable. Others available are listed in Linscott's Directory of Immunological and Biological Reagents (9th edition, 1996-7) and include, for example, an anti α -hCG monoclonal available from Biostride Inc. (CA, USA), and an anti β -hCG monoclonal available from Biogenesis Ltd (Poole, Dorset, UK).

The capture/detection of hCG takes place as follows:

- v. 200 μl of hCG (10 IU/ml) was incubated with 20 μl of a second monoclonal antibody recognising hCG (termed 3468, at 100 $\mu\text{g/ml}$) for 30 minutes at room temperature (again, antibodies other than 3468 would be suitable). This was performed in order to increase the effective mass of hCG and thus increase the sensitivity of detection.
- vi. 40 μl of the premixed hCG-3468 solution was injected across flow cell 1. This can be seen marked (a) in figure 5A.
- vii. Immediately following the injection, the buffer flow was altered to pass over the second surface after leaving the first surface. The point at which this took place is marked (b) in figure 5B.

Figure 5A shows that hCG-3468 complex dissociating from the first surface can be bound and detected at the second surface by capture with anti-hCG antibody 3299 (Fig. 5B). Those skilled in the art will appreciate that, in practice, there is no need for the first surface to generate a detectable signal - such was the arrangement in this example for the purposes of investigation only. In a normal arrangement only the capture (second) surface would give rise to a detectable signal, such that the first surface could be any suitable solid support. Moreover, in the assay method of the invention, conditions would be arranged such that hCG was displaced from surface 1 only in the presence of the analyte of interest. The example nevertheless demonstrates the principle of the invention.

Example 4

This example relates to the specific displacement of an antibody (4155) recognising estrone 3-glucuronide (E3G) from a low affinity E3G analogue (estradiol 3-glucuronide [ED3G]) at one surface and capture and detection by an antibody recognising immunoglobulin at a second surface. Figure 6 schematically illustrates this detection principle.

The 4155 monoclonal cell line was prepared and screened according to the methods described by Gani *et al.*, (1994 J. Steroid Biochem. Molec. Biol. 48, 277-282). The Gani *et al.* publication relates to development of anti-progesterone antibodies, but essentially identical techniques were employed in producing antibodies reacting with estrone and analogues thereof. Antibodies other than that obtainable from cell line 4155 may readily be produced by those skilled in the art (using such techniques): such antibodies would have qualitatively similar properties. Moreover, a commercially available anti-estrone glucuronide monoclonal antibody (from Wallaceville Animal Research Centre, New Zealand) is described in Linscott's Directory of Immunological and Biological Reagents (9th edition, 1996-7).

Initially, a CM5 sensor chip was set up as follows:

- i. A new CM5 sensor chip was docked in the Bialite™ biosensor and equilibrated with HBS running buffer. The temperature was maintained at 25°C and the flow rate set to 10 µl/min.

- 001100 071100 054956 054956
- ii. The liquid flow was set to cross a first surface ("surface 1") and a second surface ("surface 2") in series. The dextran surface was activated using EDC and NHS activating chemicals from the Biacore AB amine coupling kit by injecting the EDC/NHS mixture into the sample loop and loading 40 μ l. This can be seen marked (a) on figure 7.
 - iii. The buffer flow was set to cross only surface 2 and 40 μ l of rabbit anti-mouse IgG in sodium acetate buffer, pH 5.0 was injected. This can be seen marked (b) on figure 7.
 - iv. Following IgG coupling to surface 2 the buffer flow was changed to flow only over surface 1 and 40 μ l ethylene diamine (EDA) (20% v/v) injected. This can be seen marked (c) on figure 7.
 - v. 20 μ l of ED3G (5 mg/ml) was added to 80 μ l of a solution of NHS/EDC activation solution, mixed and incubated for 7 minutes at room temperature. 40 μ l of this preincubated ED3G solution was then injected across surface 1 (this can be seen marked (d) on figure 7).
 - vi. The buffer flow was set to flow only over surface 2 and 40 μ l of a 1M solution of ethanolamine was injected. This can be seen marked (e) on figure 7.

Displacement and recapture of 4155 was performed as follows:

- vii. 4155 was loaded onto surface 1 by the injection of 20 μ l of a 100 μ g/ml solution of 4155.
- viii. Specific displacement from surface 1 and recapture/detection at surface 2 was induced by the injection of a 1 mg/ml solution of E3G across surfaces 1 and 2 for two minutes at various flow rates. The results of this can be seen in figure 8, in which the filled boxes show the specific displacement of 4155 from surface 1 by E3G and the open boxes show the capture of 4155 at surface 2. Increasing box

size denotes an increase in flow rate where the smallest size box represents 2 $\mu\text{l}/\text{min}$ and subsequent box sizes are of data collected at 5, 10 and 20 $\mu\text{l}/\text{min}$.

The percentage of the displaced 4155 captured and detected at surface 2 is clearly related to the flow rate (see figure 9). An increase in flow rate decreases the percentage of displaced material. Low flow rates (less than 5 $\mu\text{l}/\text{min}$) can ensure all the displaced material is captured.

This example shows how analyte (E3G) can be assayed by inducing the specific displacement of a molecule reversibly bound to one surface and subsequent recapture of the displaced material at a second distinct surface by, for example, an antibody recognising the displaced molecule.

Example 5

This example relates to the specific displacement of an antibody (4101) recognising estrone 3-glucuronide (E3G) from a low affinity E3G analogue (estradiol 3-glucuronide [ED3G]) at one surface and capture by E3G at a second, detecting surface. Figure 10 schematically illustrates this detection principle.

Initially, a CM5 sensor chip was set up as follows:

- i. A new CM5 sensor chip was docked in the Bialite™ biosensor and equilibrated with HBS running buffer. The temperature was maintained at 25°C and the flow rate set to 10 $\mu\text{l}/\text{min}$.
- ii. The liquid flow was set to cross a first surface ("surface 1") and a second surface ("surface 2") in series. The dextran surface was activated using EDC and NHS activating chemicals from the Biacore AB amine coupling kit by injecting the EDC/NHS mixture into the sample loop and loading 40 μl . This can be seen marked (a) on figure 11.
- iii. 40 μl of EDA (20% v/v) was injected across surface 1 and 2. This can be seen

marked (b) on figure 11.

- iv. 20 μ l of ED3G (5 mg/ml) was added to 80 μ l of a solution of NHS/EDC activation solution, mixed and incubated for 7 minutes at room temperature. 40 μ l of this preincubated ED3G solution was then injected across surface 1 (this can be seen marked (c) on figure 11).
- v. 20 μ l of E3G (5 mg/ml) was added to 80 μ l of a solution of NHS/EDC activation solution, mixed and incubated for 7 minutes at room temperature. 40 μ l of this preincubated E3G solution was then injected across surface 2 (this can be seen marked (d) on figure 11).

Specific displacement and capture of 4101 was performed as follows:

- vi. 4101 was loaded onto surface 1 by the injection of 20 μ l of a 100 μ g/ml solution of 4101 across surface 1.
- vii. Displacement of 4101 from surface 1 and recapture/detection at surface 2 was induced by the injection of 20 μ l of a 1 mg/ml solution of estrone 3-sulphate [E3S] across surface 1 and 2. The displacement from surface 1 and capture/detection at surface 2 can be seen in figure 12.

In this example E3S represents the analyte. This example shows how analyte can be assayed by inducing the displacement of a molecule reversibly bound to a lower affinity analogue of the analyte (ED3G) at one surface and subsequently recaptured and detected at a second distinct surface by binding to a higher affinity analogue of the analyte (E3G).

Example 6

This example shows the production of a colour change brought about by the displacement/capture of a glucose, oxidase/antibody conjugate in response to analyte addition. The displaced glucose oxidase/antibody conjugate is captured on a thin layer of

starch impregnated with KI, horse radish peroxidase and a capture molecule. The captured glucose oxidase/antibody conjugate utilises glucose to generate H_2O_2 , which oxidises iodide to iodine in the presence of peroxidase. The iodine generated then reacts with starch, resulting in a colour change from brown to blue.

Two surfaces are set up as follows:

First Surface ("Surface 1")

- i. An estradiol 3-glucuronide (ED3G)/ovalbumin conjugate is prepared by resuspending 2.6 mg ED3G in 2 ml of a freshly prepared solution of EDC (0.1 M) and NHS (0.02 M) and incubating for 15 min at room temperature.
- ii. To the ED3G solution, 2 ml ovalbumin (10 mg/ml) is added and this is incubated for 2.5 h at room temperature with constant mixing.
- iii. The conjugated ED3G/ovalbumin solution is then dialysed for 16 h against 1L phosphate buffered saline containing 0.1% sodium azide ("PBSA").
- iv. Griener HB wells are coated with the dialysed ED3G-ovalbumin conjugate by incubating 100 μ l ED3G-ovalbumin (diluted 1 in 10 with PBSA) in the wells for 2 h at room temperature.
- v. A conjugate of glucose oxidase and monoclonal antibody 4155 is prepared by mixing 1ml glucose oxidase in 100 mM phosphate buffer pH 6.5 with 1 ml of monoclonal antibody 4155 (also in 100 mM phosphate buffer pH 6.5) in a glass tube at a protein concentration of 5 mg/ml. To this 220 μ l of 0.02% glutaraldehyde is added dropwise with constant mixing. The tube is wrapped in aluminium foil and mixed end over end for 30 min at room temperature. 250 μ l of 1 M ethanolamine is added and mixing continued for a further 30 min. The solution is then dialysed against 100 mM PBS at room temperature for 24 h then centrifuged at 10,000 rpm for 30 min at 4°C.

- vi. The Griener HB surface is then primed by incubating 100 μ l glucose oxidase-4155 conjugate per well for 1 h at room temperature. Wells are then rinsed with PBS to remove unbound glucose oxidase/4155 conjugate. Wells are stored containing 100 μ l PBS at 4°C until required.

Second Surface ("Surface 2")

- i. A 200 μ l solution containing 100 μ l estrone 3-glucuronide (E3G)-ovalbumin conjugate (prepared as described for the ED3G-ovalbumin conjugate), 5% (w/v) starch, 10 mg/ml horseradish peroxidase and 0.1 M KI in PBS is incubated in a Griener HB well for 16 h at room temperature. The surface is brown in colour.

Analyte (1 ml estrone 3 glucuronide at 0.1 mM) in a 10 mM glucose solution in PBS is pumped at 0.2 ml/min into the well with the primed surface 1 and at the same time solution from this well is pumped at 0.2 ml/min into the well containing surface 2. In order that the well with surface 2 does not overflow, solution is also pumped out of the well at 0.2 ml/min.

Upon starting the flow of test solution from surface 1 to surface 2 the colour of surface 2 changes from brown to blue as the iodide is oxidised to iodine by the H_2O_2 generated by the oxidation of glucose by glucose oxidase.

Example 7

This example relates to the modulation of fluorescence intensity at a surface in response to analyte. Upon challenge with analyte, molecules are displaced from a first surface. These displaced molecules are then able to modulate the intensity of fluorescence generated at a second surface.

Two surfaces are set up as follows:

First Surface ("Surface 1")

- i. An estradiol 3-glucuronide (ED3G)/ovalbumin conjugate is prepared by resuspending 2.6 mg ED3G in 2 ml of a freshly prepared solution of EDC (0.1 M) and NHS (0.02 M) and incubating for 15 min at room temperature.
- ii. To the ED3G solution 2 ml ovalbumin (10 mg/ml) is added and this is incubated for 2.5 h at room temperature with constant mixing.
- iii. The conjugated ED3G/ovalbumin solution is then dialysed for 16 h against 1L PBSA.
- iv. Griener HB wells are coated with the dialysed ED3G/ovalbumin conjugate by incubating 100 μ l ED3G/ovalbumin (diluted 1 in 10 with PBSA) in the wells for 2 h at room temperature.
- v. A conjugate of cellulose binding domain (CBD)/monoclonal antibody 4155 is prepared by mixing 1 ml CBD in 100 mM phosphate buffer pH 6.5 with 1 ml of monoclonal antibody 4155 (also in 100 mM phosphate buffer pH 6.5) in a glass tube at a protein concentration of 5 mg/ml. To this 220 μ l of 0.02% glutaraldehyde is added dropwise with constant mixing. The tube is wrapped in aluminium foil and mixed end over end for 30 min at room temperature. 250 μ l of 1 M ethanolamine is added mixing continued for a further 30 min. The solution is then dialysed against 100 mM PBS at room temperature for 24 h then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant is stored at 4°C until required.
- vi. The Griener HB surface is then primed by incubating 100 μ l CBD-4155 conjugate per well for 1 h at room temperature. Wells are then rinsed with PBSA to remove unbound CBD/4155 conjugate.

Second Surface ("Surface 2")

- i. A 1 ml solution of H₃PO₄swollen Avicel (0.2%) is incubated with 50 μ l locust bean gum (LBG)-FITC conjugate for 1h with constant mixing at room temperature.

- ii. LBG-FITC Avicel is washed by subjecting the particles to centrifugation at 1300 rpm for 10 min at room temperature and resuspending the pellet in PBS. This wash procedure is repeated a second time.
- iii. Following the second wash the LBG-FITC loaded particles are resuspended in 50 μ l PBS and stored at 4°C until required.

Analyte (100 μ l E3G at 1 mg/ml) is incubated for 1 h in primed Griener HB wells (surface 1) at room temperature. The incubated solution is then removed from the wells and added to LBG-FITC loaded particles. A decrease in fluorescence intensity due to the displacement of LBG-FITC by CDB-4155 on the Avicel particles is monitored using a fluorescent activated cell sorter, or by a cell sorter (e.g. the Coulter Elite™ cell sorter).

Example 8

This example shows the production of an electrical change brought about by the displacement/capture of a bispecific antibody fragment construct in response to analyte addition. It relates to the specific displacement of the first antibody binding site (recognising estrone-3-glucuronide (E3G) and derived from mouse monoclonal antibody 4155) from a low affinity E3G analogue (estradiol 3-glucuronide (ED3G)) from a first surface, and capture and detection through the second antibody binding site recognising an electroactive molecule immobilised at a second surface. The second surface is a gold electrode coated with a monolayer of the electroactive molecule. The electroactive molecule is a carbazole residue, coupled to a hexyl pendant portion, which is in turn coupled to the gold surface by a thiol group. Our co-pending European Patent application, filed on even date herewith, describes how the binding of selected antibodies can alter (modulate) the electrical current flowing through the electrode under the influence of an applied voltage. Here the binding of the bispecific antibody fragment to the electroactive molecule thus modulated the current flow in a manner which was directly related to the concentration of analyte originally introduced.

The 4155 monoclonal cell line was prepared and screened according to methods described by Gani *et al*, (1994 J. Steroid Biochem. Molec. Biol. **48**, 277-282). The Gani *et al* publication relates to the development of anti-progesterone antibodies, but essentially identical techniques were employed in producing antibodies reacting with estrone and analogues thereof. Antibodies other than that obtainable from cell line 4155 may readily be produced by those skilled in the art (using such techniques): such antibodies would have qualitatively similar properties. Monoclonal antibodies against the electroactive carbazole molecule, another hapten, can also be produced using these methods. The cloning of the genes for the two antibody binding specificities and their use in the construction of a bispecific antibody fragment can easily be accomplished by methods well known to those skilled in the art; (see for example WO 97/14719).

Two surfaces are set up as follows:

First surface ("Surface 1")

- i. An estradiol 3-glucuronide (ED3G)/ovalbumin conjugate is prepared by resuspending 2.6mg of ED3G in 2ml freshly prepared solution of EDC (0.1M) and NHS (0.02M) and incubating for 15 mins at room temperature.
- ii. To the ED3G solution, 2ml ovalbumin (10mg/ml) is added and this is incubated for 2.5hr at room temperature with constant mixing.
- iii. The conjugated ED3G/ovalbumin solution is then dialysed for 16hr against 1L phosphate buffered saline containing 0.1% sodium azide ("PBSA").
- iv. Greiner HB wells are coated with dialysed ED3G-ovalbumin conjugate by incubating 100 μ l ED3G-ovalbumin (diluted 1 in 10 with PBSA) in the wells for 2 hr at room temperature.
- v. The Greiner HB-ED3G-ovalbumin surface is then primed by incubating 100 μ l of a solution of the bispecific antibody fragment (100 μ g/ml in PBSA) per well for 1 hr at room

temperature. Wells are then rinsed with PBSA to remove unbound bispecific antibody fragment. Wells were stored containing 100 μ l of PBSA at 4°C until required.

Second surface ("Surface 2")

- i. The thiol terminus alkyl pendant electroactive (carbazole) molecule was dissolved in nitrogen purged ethanol or DMF solution.
- ii. A clean pure gold surface was dipped into the solution containing the electroactive moiety for 24hr in the dark.
- iii. The electroactive monolayer on the gold surface was washed in a suitable solvent (e.g. dichloromethane) to remove any unbound molecules and then stored overnight in a suitable solvent that has been nitrogen purged.
- iv. The electrode was dried in a stream of nitrogen to remove solvent from the electrode surface.
- v. The electrode was transferred through a series of increasingly dilute acetonitrile: water mixes to improve the monolayer's molecular orientation, which results in a monolayer which is stable in an aqueous environment.

Assay

Electrochemical measurements are performed by cyclic voltammetry or chronoamperometry on a Princeton Applied Research Corporation Scanning Potentiostat (model 362) together with a Bryan flat bed X-Y recorder (model A25000) and a EH&G model 273A Princeton Applied Research Potentiostat/Galvanostat (using Echem and Lotus 123 to process the data).

- (a) The background electrochemistry is read and stored once a stable scan had been produced.

- (b) The sample to be read is added to surface 1 (the well) and incubated for 15 minutes at room temperature.
- (c) The sample is removed from the well and added to surface 2 (the electrode surface), and again incubated at room temperature for 15 minutes.
- (d) The electrode surface is washed with MilliQ water and the electrochemistry read using the same conditions as (b) above. The modulation of the current flow gives a measure of the analyte concentration which may be expressed in molar concentration units if the system has been calibrated against standard E3G samples.

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CLAIMS

1. A method of detecting the presence of an analyte of interest in a sample, the method comprising the steps of: providing a first surface having reversibly immobilised thereon a displaceable moiety; exposing the first surface to a sample comprising the analyte of interest, the analyte of interest specifically displacing the displaceable moiety from the first surface in an affinity-related manner; causing the displaceable moiety displaced from the first surface to contact a second surface bearing a capture moiety which specifically binds to the displaceable moiety, so as to capture the displaceable moiety on the second surface, said capture generating a detectable signal; and detecting the signal; wherein said detection is performed by means other than SPR, and wherein the displaceable moiety cannot generate the signal which is detected in the assay unless and until the displaceable moiety is captured on the second surface.
2. A method according to claim 1, wherein the displaceable moiety comprises an immunoglobulin molecule or an antigen-binding derivative thereof.
3. A method according to claim 1 or 2, wherein the displaceable moiety comprises a bispecific antibody or bispecific antigen-binding antibody derivatives.
4. A method according to any one of claims 1, 2 or 3, wherein the displaceable moiety comprises a portion which facilitates reversible immobilisation on the first surface.
5. A method according to any one of the preceding claims, wherein the displaceable moiety comprises a fusion protein.
6. A method according to any one of the preceding claims, wherein the displaceable moiety comprises a mimotope which is an analogue of the analyte of interest.
7. A method according to any one of the preceding claims, wherein the first surface comprises a plurality of intervening molecules which bind relatively loosely to the displaceable moiety, such that the binding affinity of the intervening moiety for the analyte

of interest is greater than that of the displaceable moiety for the intervening moiety.

8. A method according to claim 8, wherein the intervening molecule is an analogue (e.g. mimotope) of the analyte of interest.
9. A method according to any one of the preceding claims, wherein the capture moiety comprises an immunoglobulin molecule or an antigen-binding variant thereof.
10. A method according to any one of the preceding claims, wherein the displaceable moiety and the capture moiety comprise the members of a specific binding pair.
11. A method according to any one of the preceding claims, wherein the detectable signal comprises the generation of, or the modulation of, an evanescent or acoustic wave.
12. A method according to any one of claims 1-10, wherein capture of the displaceable moiety by the capture moiety directly modulates the electrochemical properties of the capture moiety, which modulation comprises the detectable signal.
13. A method according to any one of the preceding claims, wherein the first and second surfaces are provided on separate respective first and second supports.
14. A method according to any one of claims 1-12, wherein the first and second surfaces are provided on a single support.
15. A method according to any one of the preceding claims, wherein the first and/or second surface is provided on a solid support which is planar, particulate or porous.
16. A method according to any one of the preceding claims, wherein the analyte of interest is selected from the group consisting of: steroid hormones, protein hormones, nucleic acids, peptides, bacterial or viral antigens, and immunoglobulins.
17. Apparatus for performing a method according to any one of the preceding claims, the

apparatus comprising: a first surface having reversibly immobilised thereon a displaceable moiety, wherein the displaceable moiety is displaced from the first surface in the presence of an analyte of interest; and a second surface bearing a capture moiety which specifically binds to the displaceable moiety;

18. Apparatus according to claim 17, further comprising one or more of the following: fluid conducting means; signal detection means for detecting a signal generated by capture of the displaceable moiety by the capture moiety; and data processing means.

19. Apparatus according to claim 17 or 18, comprising filter means provided between the first and second surfaces.

20. A method substantially as hereinbefore described and with reference to the accompanying drawings.

21. Apparatus substantially as hereinbefore described and with reference to the accompanying drawings.

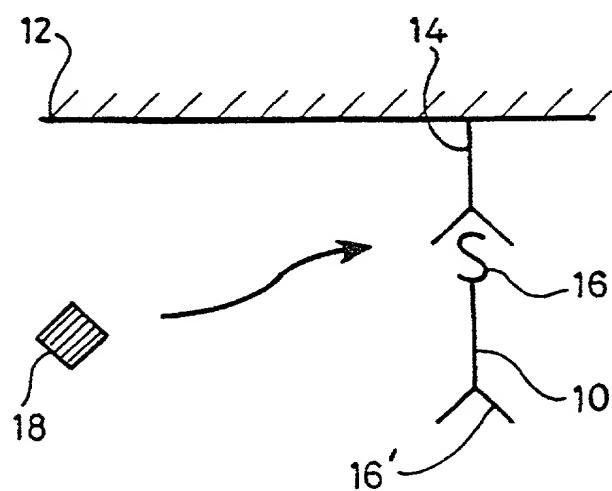
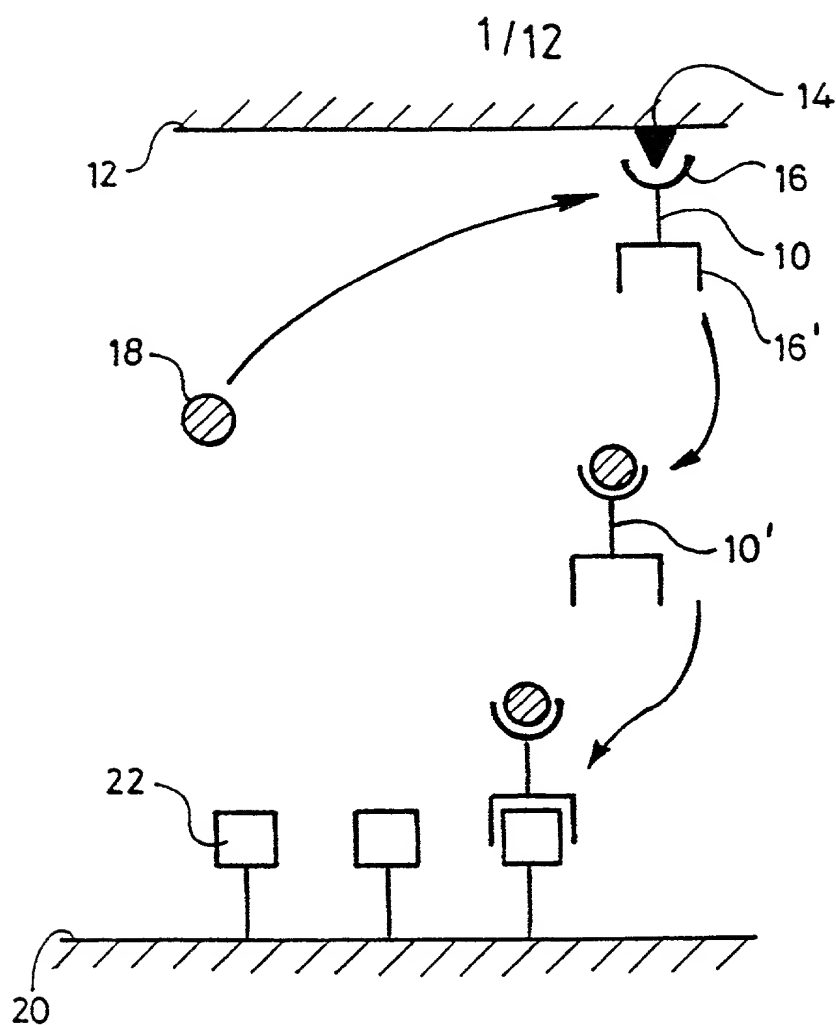


Fig. 1

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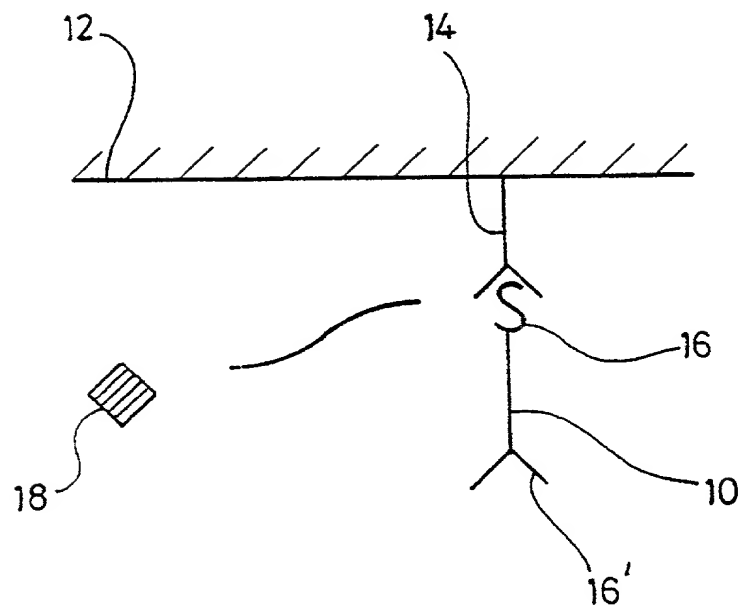


Fig. 2

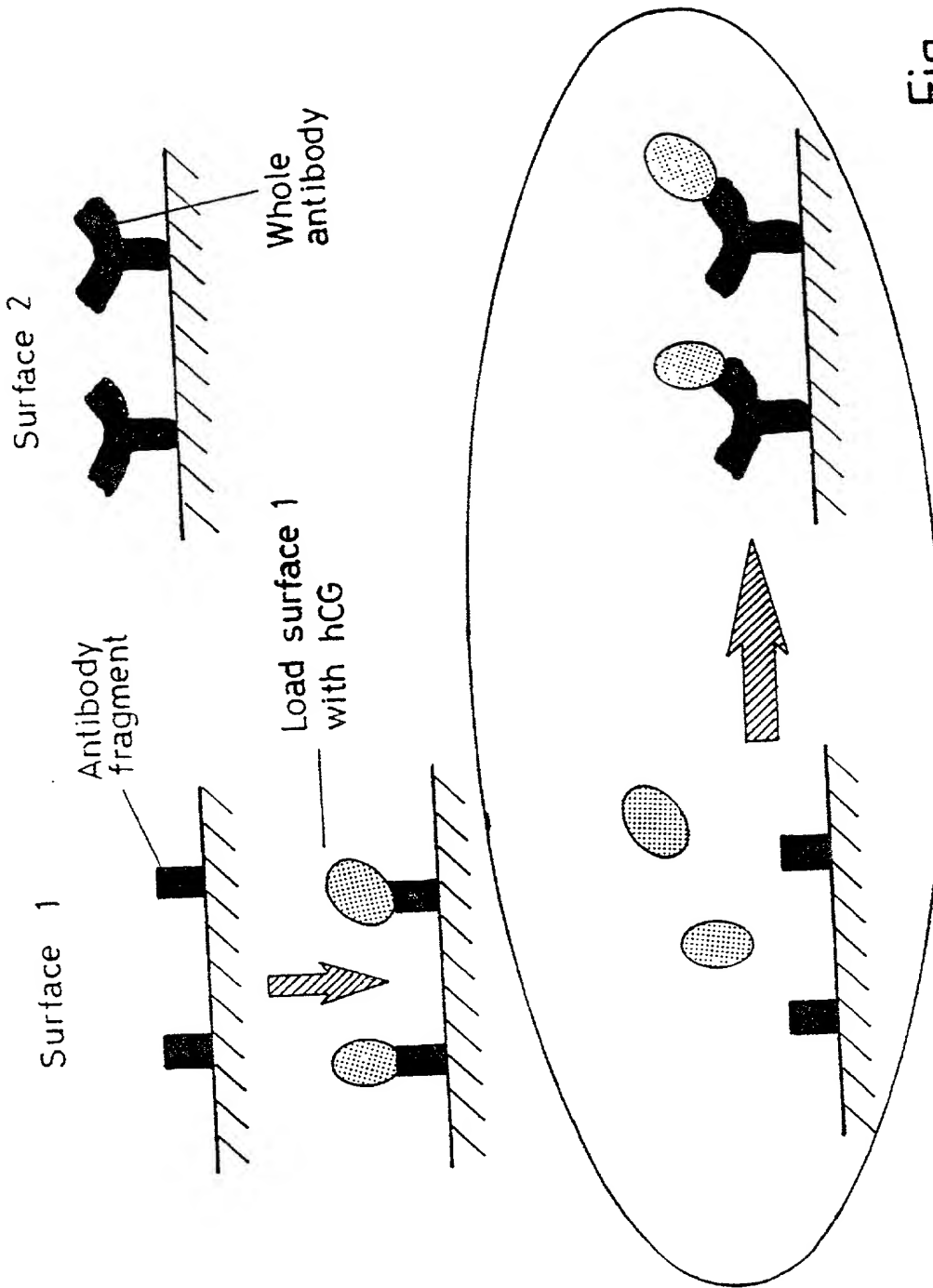


Fig. 3

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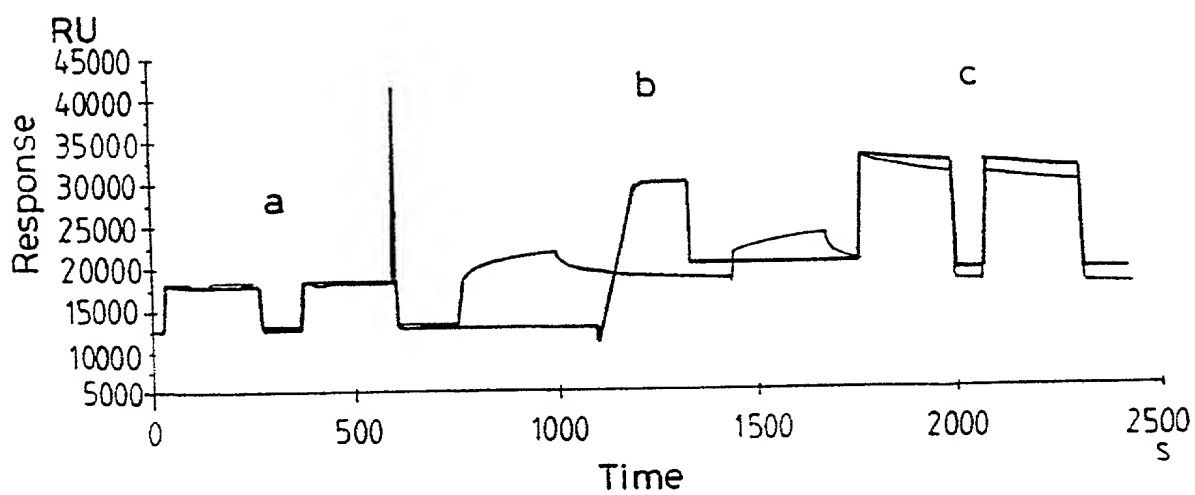


Fig. 4

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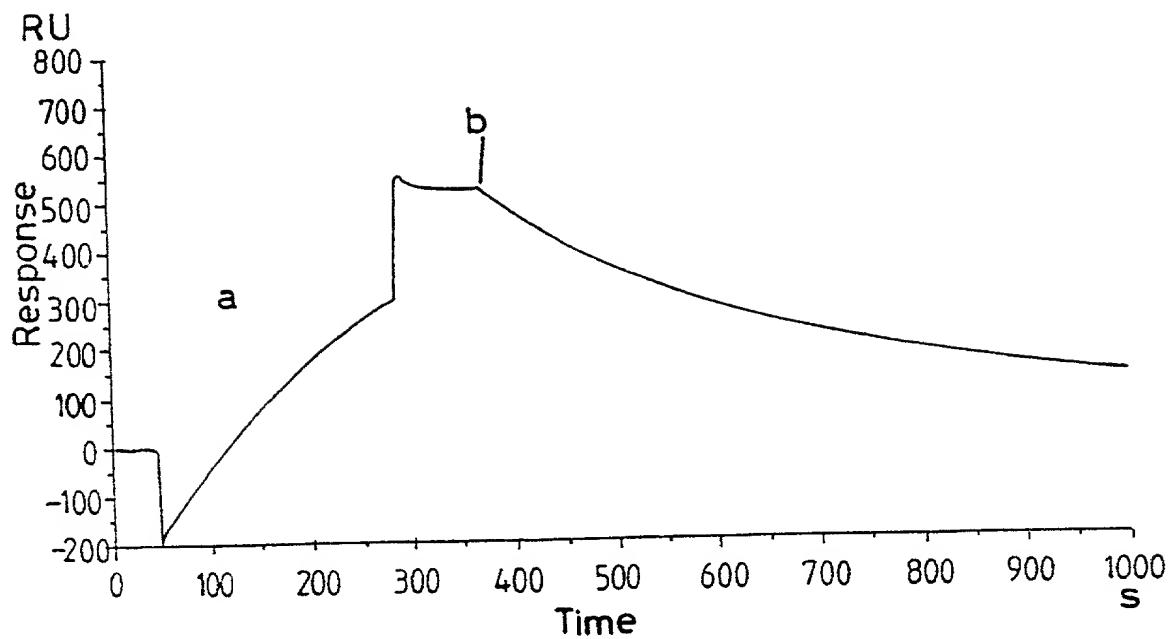


Fig. 5A

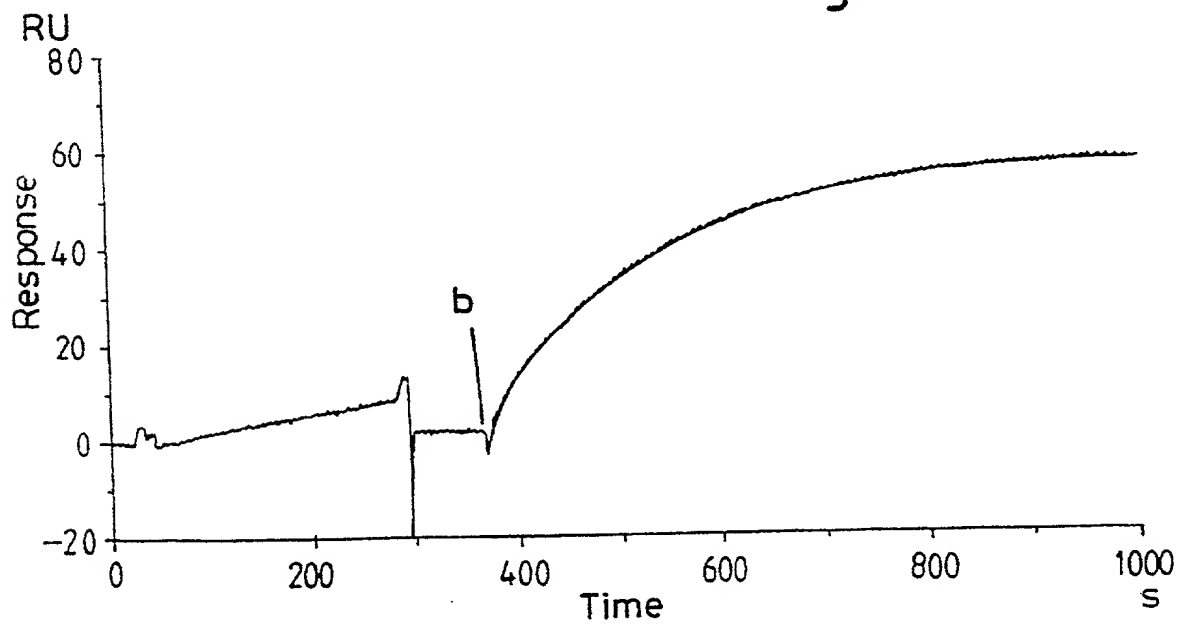


Fig. 5B

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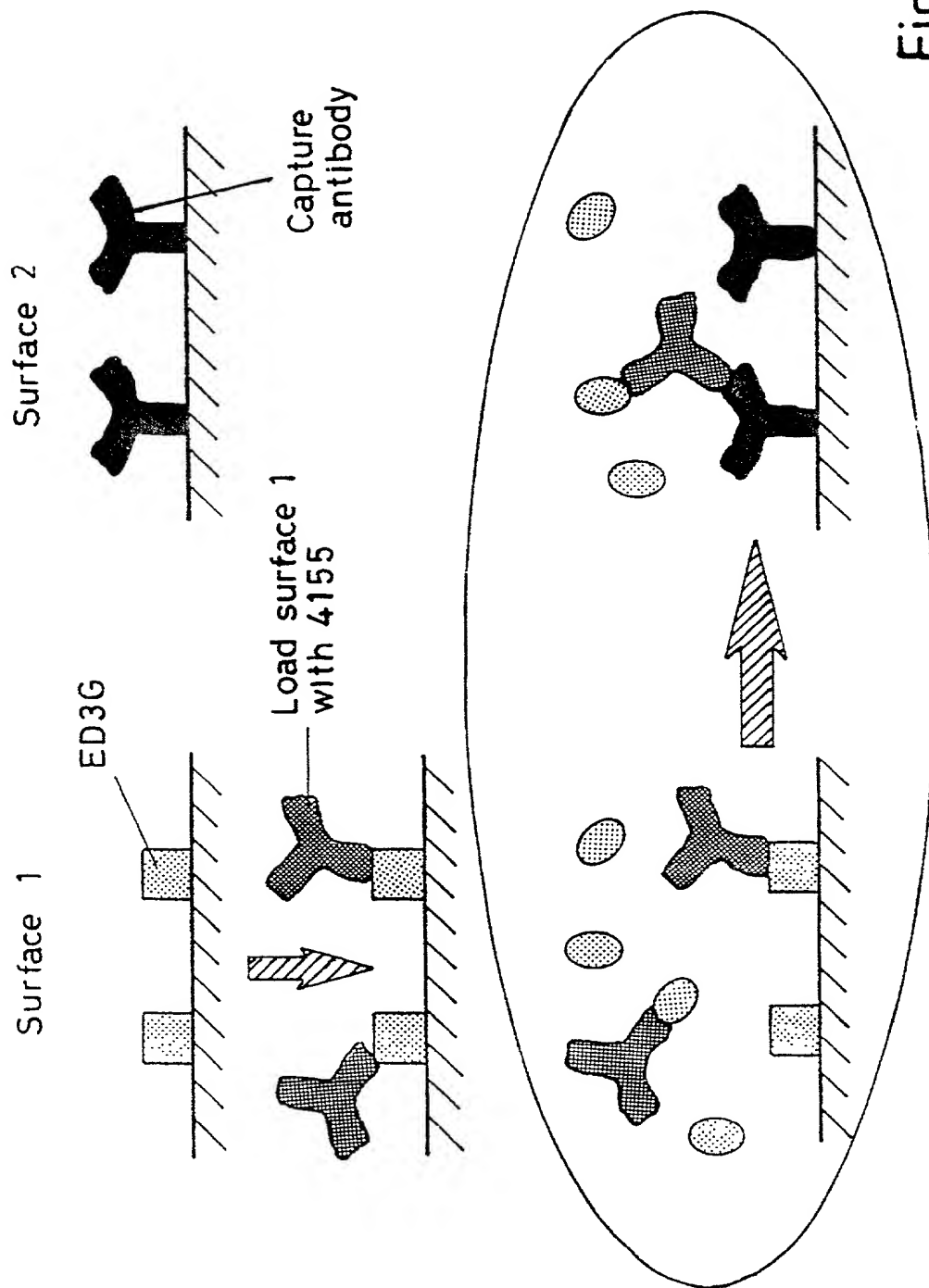


Fig. 6

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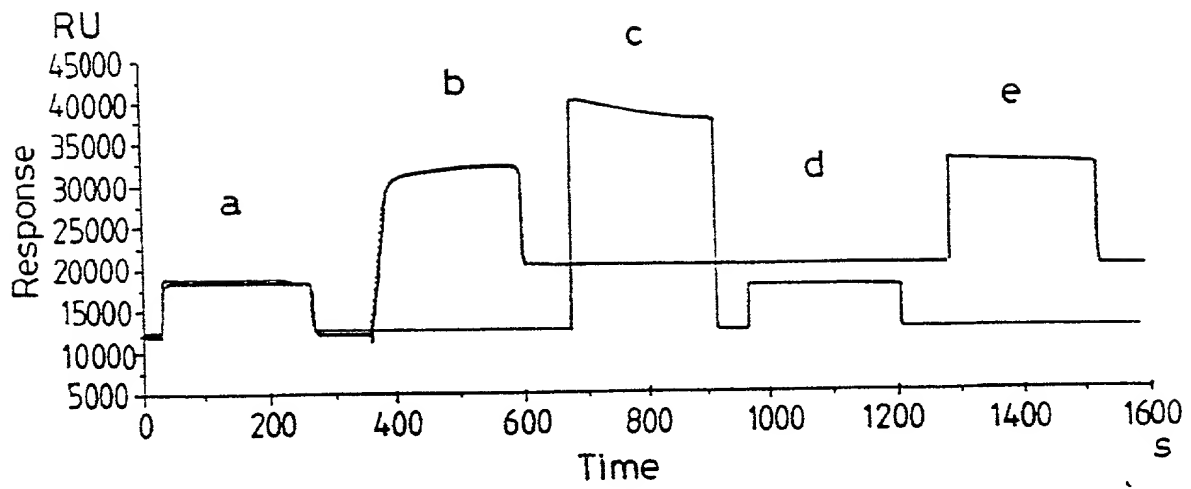


Fig. 7

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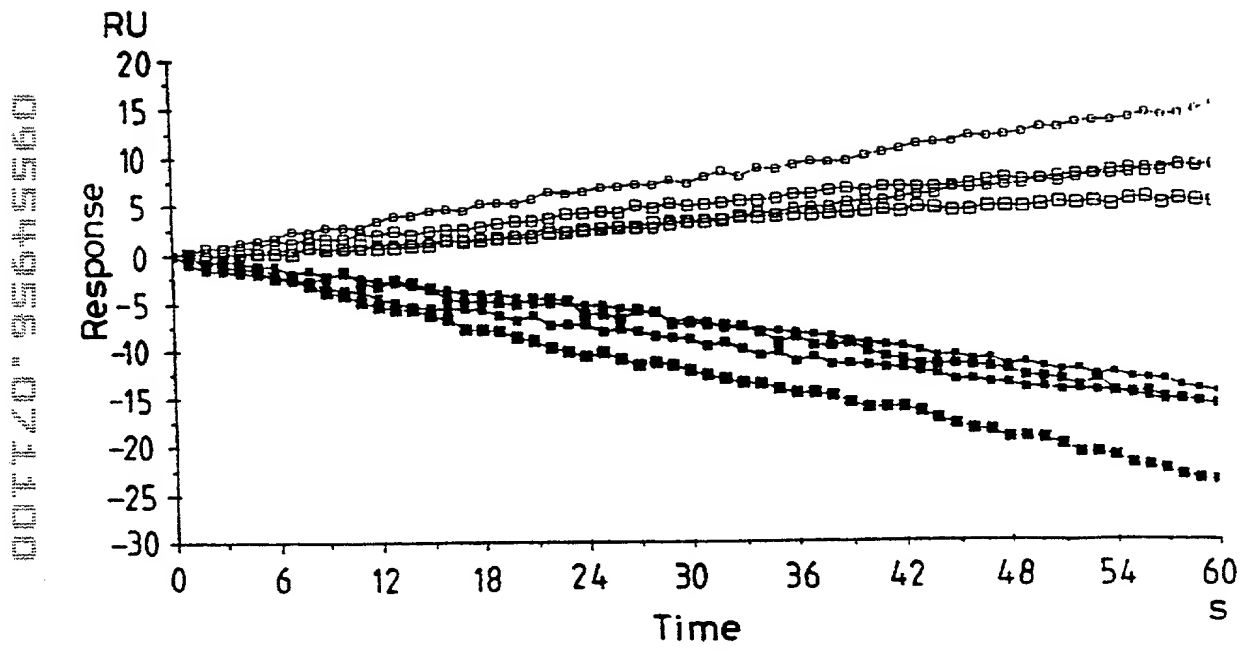


Fig. 8

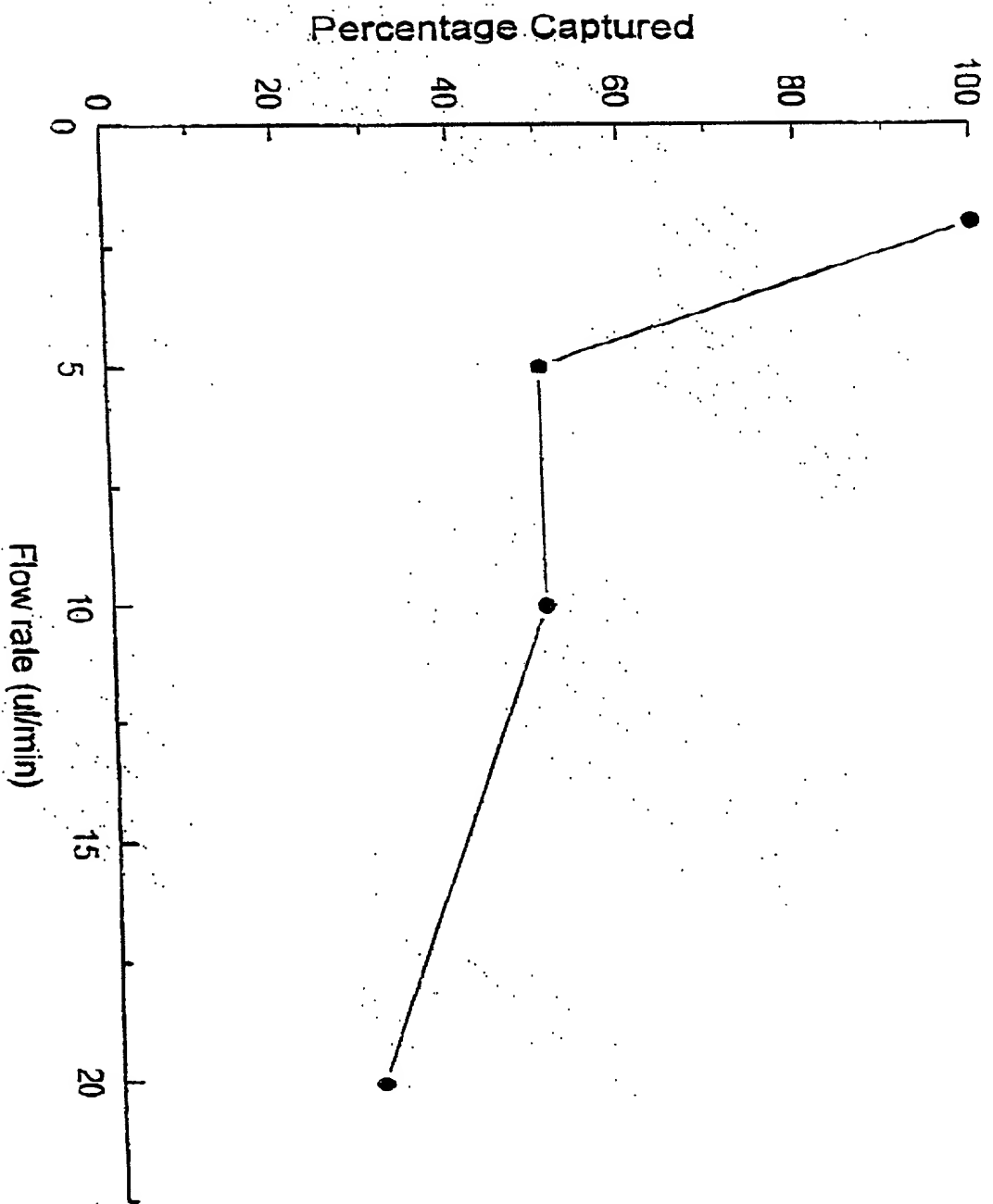


Fig. 9

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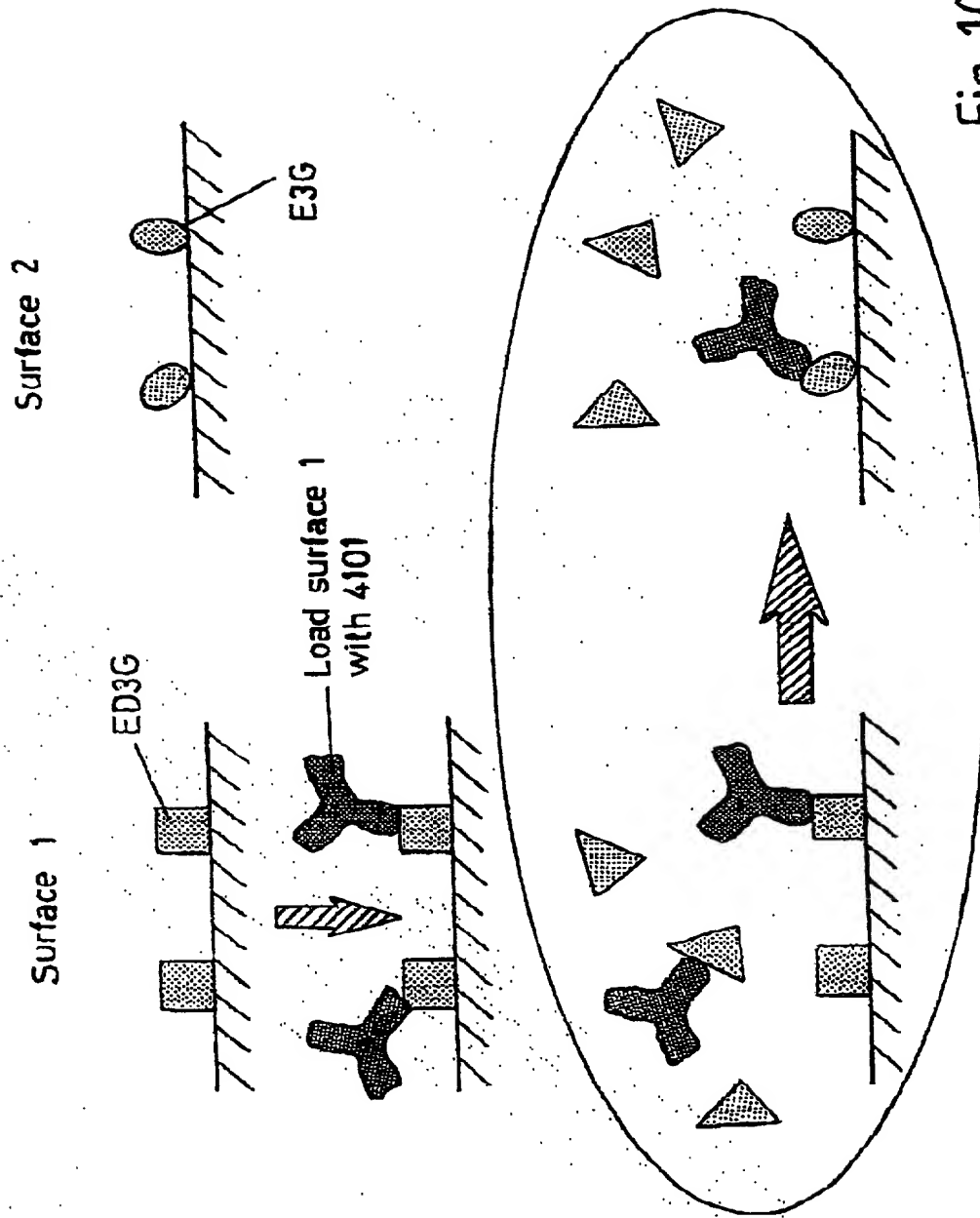


Fig. 10

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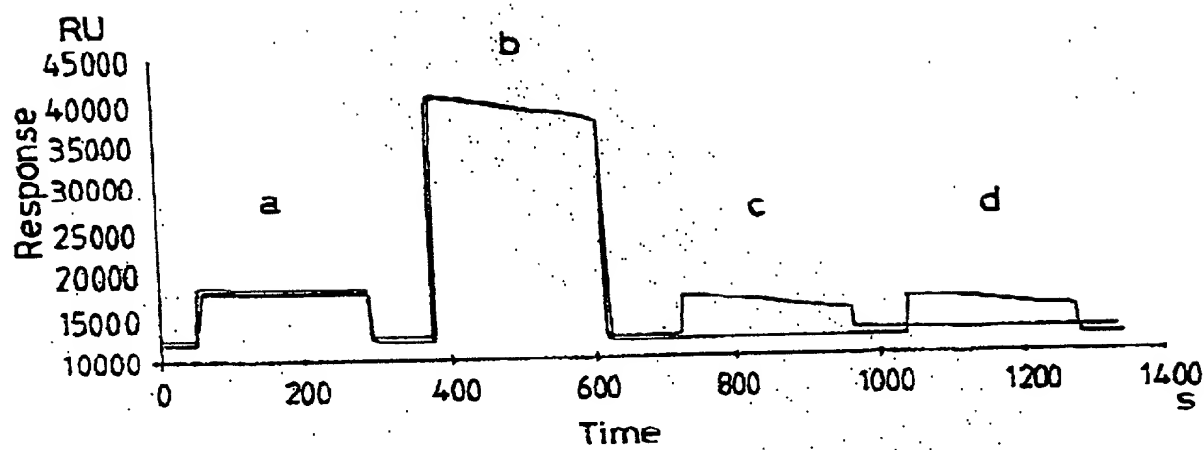


Fig. 11

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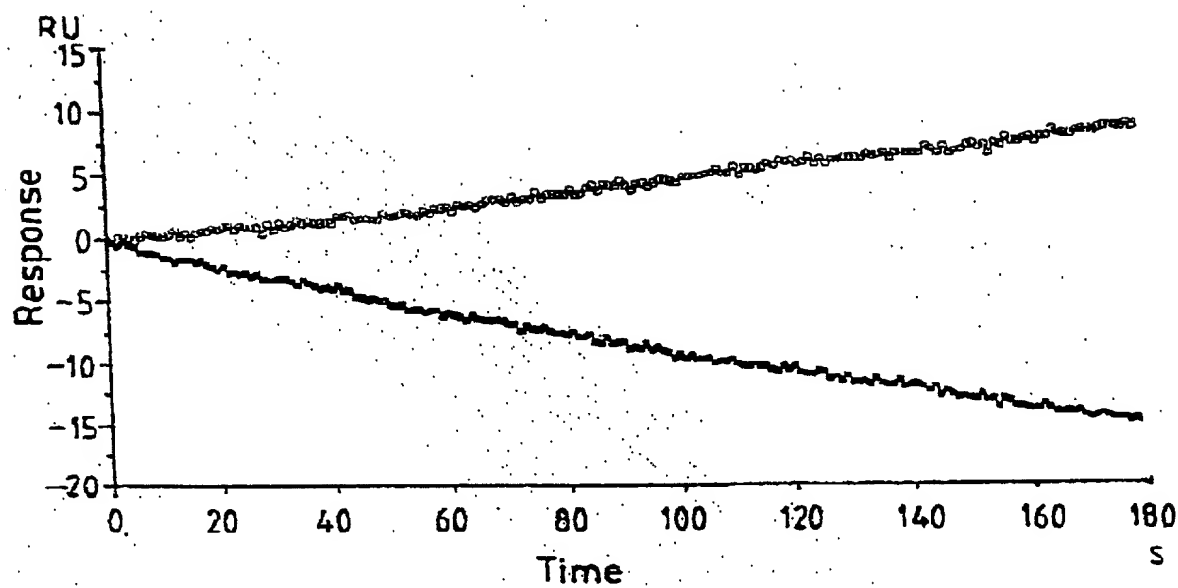


Fig. 12

Rule 56(a) & (b) = 37 C.F.R. 1.56(a) & (b)
PATENT AND TRADEMARK CASES - RULES OF PRACTICE
DUTY OF DISCLOSURE

- (a) ...Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the [Patent and Trademark] Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability... (b) information is material to patentability when it is not cumulative and (1) It also establishes by itself, or in combination with other information, a prima facie case of unpatentability of a claim or (2) refutes, or is inconsistent with, a position the applicant takes in: (i) Opposing an argument of unpatentability relied on by the Office, or (ii) Asserting an argument of patentability

PATENT LAWS 35 U.S.C.

§102. Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless--

- (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent or
- (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States, or
- (c) he has abandoned the invention, or
- (d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months* before the filing of the application in the United States, or
- (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or
- (f) he did not himself invent the subject matter sought to be patented, or
- (g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other.

§103. Condition for patentability; non-obvious subject matter

- (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made. . . .
- (c) Subject matter developed by another person, which qualified as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

* Six months for Design Applications (35 U.S.C. 172).

**FOR UTILITY/DESIGN
CIP/PCT NATIONAL/PLANT
ORIGINAL/SUBSTITUTE/SUPPLEMENTAL
DECLARATIONS**

**RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

**PM & S
FORM**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the INVENTION ENTITLED IMPROVEMENTS IN OR RELATING TO DISPLACEMENT ASSAYS

the specification of which (CHECK applicable BOX(ES))
☒ BOX(ES) ☐ A. ☐ is attached hereto.
☐ B. ☐ was filed on _____ as U.S. Application No. _____
☒ C. ☒ was filed as PCT International Application No. PCT/ GB98/03483 on November 23, 1998
 and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S)

Number	Country	Day/MONTH/Year Filed	Date first Laid-open or Published	Date Patented or Granted	Priority NOT Claimed
97309409.7	EUROPE	21 November 1997			

If more prior foreign applications, X box at bottom and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U.S.C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S)

Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status	Priority NOT Claimed
PCT/GB98/03483	23 November 1998	pending, abandoned, patented	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Madison & Sutro LLP, Intellectual Property Group, 1100 New York Avenue, N.W., Ninth Floor, East Tower, Washington, D.C. 20005-3918, telephone number (202) 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete names/numbers below of persons no longer with their firm and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above firm and/or a below attorney in writing to the contrary.

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Carl G. Love	18781	Kendrew H. Colton	30368	Richard H. Zaitlen	27248	Paul L. Sharer	36004
Kevin E. Joyce	20508	G. Paul Edgell	24238	Roger R. Wise	31204		
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Peter W. Gowdey	25872	David A. Jakopin	32995	W. Patrick Bengtsson	32456		

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FOR ADDITIONAL INVENTORS, "X" box ☒ and proceed on the attached page to list each additional inventor.
☐ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. PM 270584
 (M#)

DECLARATION AND POWER OF ATTORNEY

(continued)

ADDITIONAL INVENTORS:

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(6) INVENTOR'S SIGNATURE:

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Residence			
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Post Office Address (include Zip Code)			

(7) INVENTOR'S SIGNATURE:

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First	Middle Initial	Family Name	
Residence			
City	State/Foreign Country		Country of Citizenship
Post Office Address (include Zip Code)			

(8) INVENTOR'S SIGNATURE:

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(9) INVENTOR'S SIGNATURE:

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City	State/Foreign Country		Country of Citizenship
Post Office Address (include Zip Code)			